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In vivo biological activity of rocket extracts (*Eruca vesicaria* subsp. *sativa* (Miller) Thell) and sulforaphane

M. Villatoro-Pulido [a,*], R. Font [b], S. Saha [c], S. Obregón-Cano [d], J. Anter [e], A. Muñoz-Serrano [e], A. De Haro-Bailón [d], A. Alonso-Moraga [e], M. Del Río- Celestino [b]

[a] *IFAPA, Centro-Alameda del Obispo, Córdoba, Spain*
[b] *IFAPA, Centro La Mojonera, Almería, Spain*
[c] *Phytochemicals and Health Programme, Institute of Food Research, Norwich Research Park, NR4 TUA Norwich, United Kingdom*
[d] *Plant Breeding Department, Institute of Sustainable Agriculture, Spanish Council for Scientific Research (CSIC), Alameda del Obispo s/n, 14080 Córdoba, Spain*
[e] *Genetics Department, Campus Universitario de Rabanales, University of Córdoba, Spain*

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ABSTRACT

Eruca is thought to be an excellent source of antioxidants like phenolic compounds, carotenoids, glucosinolates and their degradation products, such as isothiocyanates. Sulforaphane is one of the most potent indirect antioxidants of *Eruca* isolated until the date. In this work we investigate: (i) the safety and DNA protective activity of *Eruca* extracts and sulforaphane (under and without oxidative stress) in *Drosophila melanogaster*; and (ii) the influence on *D. melanogaster* life span treated with *Eruca* extracts and sulforaphane. Our results showed that among the four concentrations of *Eruca* extracts tested (from 0.625 to 5 mg/ml), intermediate concentrations of the Es2 accession (1.25 and 2.5 mg/ml) exhibited no genotoxic activity, as well as antigenotoxic activity (inhibition rate of 0.2–0.6) and the lowest concentration of Es2 and Es4 accessions (0.625 mg/ml) also enhanced the health span portion of the live span curves. Sulforaphane presented a high antigenotoxic activity in the SMART test of *D. melanogaster* and intermediate concentrations of this compound (3.75 μM) enhanced average healthspan. The results of this study indicate the presence of potent antigenotoxic factors in rocket, which are being explored further for their mechanism of action.

1. Introduction

Food consumption plays an important role modulating some degenerative processes that influence the quality of life of an organism (Boyd et al., 2011). The strongest evidence that vegetables and fruits are related to a potential reduction in cancer risk comes from epidemiological studies for cruciferous vegetables (Gasper et al., 2007). Among vegetables, species of cruciferous like rocket (*Eruca vesicaria* subsp. *sativa*) contain a range of health-promoting phytochemicals including carotenoids, vitamin C, fibres, polyphenols, and glucosinolates (GLs).

GLs are hydrolyzed by myrosinases into different degradation products with a variety of biological activities (Chen and Andreasson, 2001). Some degradation products are involved in plant nutrition (Kutz et al., 2002) and growth regulation (Grubb et al., 2004). From a human perspective, glucosinolate metabolites influence human health and the potential utility of plants. It has been speculated that the isothiocyanates (ITCs) like sulforaphane (4-methylsulfinylbutyl), obtained from hydrolysis of GLs, are in great part responsible for the protective effects of cruciferous vegetables (Mithen, 2001; Juge et al., 2007). SF is the most investigated ITC *in vivo* and *in vitro* and it is derived from the GL glucoraphanin (4-methylsulfinyl butyl). Glucoraphanin and sulforaphane afford cardiovascular protection via their antioxidant and anti-inflammatory properties, resulting in reduced oxidative stress, improvement in lipid profiles, and decreased blood pressure. Other clinical trials demonstrated the bacteriocidal (Galan et al., 2004) and chemoprotective properties of sulforaphane in individuals with *H. pylori* infection (Yanaka, 2005).

Previous studies have revealed that the concentrations required for the ITCs to exert the observed effects are in the low micromolar range (30 mol/L in most cases). Higher ITC concentrations appeared to cause their anticarcinogenic activities to disappear. On the other hand, since ITCs are electrophilic and elevate reactive oxygen species, excessive doses of these compounds can be potentially toxic (Rose et al., 2003).

Abbreviations: GL, glucosinolate; ITC, isothiocyanate; SF, sulforaphane.

* Corresponding author.
E-mail addresses: myriamvillatoro@hotmail.com, myriamvillatoro@gmail.com (M. Villatoro-Pulido).

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The degenerative processes conducting to senescence are a multifaceted events caused by a gradual decline in physiological function and an increased incidence of various diseases, including cancer, neurodegenerative diseases, and diabetes (Fontana et al., 2010; Kenyon et al., 2010). A prime candidate of senescence has been the damage caused by the reactive oxygen species (ROS), which are endogenous molecular species generated primarily during respiration of the cell (Harman, 1956; Kenyon, 2010). This theory named as the free radical theory of ageing (Harman, 1956), states that the cumulative damage by oxygen free radicals is the major driver of ageing. It has also been proposed that increasing antioxidant defence should decrease steady-state levels of oxidative damage, which would then increase life span (Lustgarten et al., 2011). Evidence also suggests that transformed cells use ROS signals to drive proliferation and other events required for tumour progression. This confers a state of increased basal oxidative stress to the transformed cells (Schumacker, 2006). It seems that anticancer therapy is frequently efficient in these early stages of the disease (Benhar et al., 2002).

The extensive knowledge of the genetics of *Drosophila melanogaster* and the long experimental experience with this organism has made it useful in genetic toxicology (Graf et al., 1984, 1998). The Somatic Mutation And Recombination Test (SMART) in proliferating wings imaginal discs of *D. melanogaster* is based on the loss of heterozygosity for two genetic markers that affect the phenotype of wing hairs. There is a wide variety of compounds and complex mixtures that have been assayed with the SMART model test, such as food additives, beverages and insecticides (Yeh and Yen, 2005, Romero-Jiménez et al., 2005; Villatoro-Pulido et al., 2009).

Anti-ageing and anti-degenerative assays can be carried out using different *D. melanogaster* strains in order to perform life span trials with specific chronic diets in controlled environments (Fleming et al., 1992). In this work we describe a study designed to examine the effects of rocket extract and SF supplementation in the diet on life span in *D. melanogaster*. This animal model is an excellent system to investigate the longevity-promoting properties of compounds and nutraceutical extracts. *Drosophila* has a short life span, can be cultured with simple diets, and represents a rich genetic resource with a fully sequenced genome, and, more importantly, over half of the fly genes have mammalian homologs (Boyd et al., 2011; Jones and Grotewiel, 2011). With the evidences of the protective role of cruciferous vegetables and the compounds that they contain described previously we also have hypothesised that the treatment with SF and/or *Eruca* extracts may enhance the *Drosophila* life span.

With the background evidences of the protective effects of SF and cruciferous vegetables we have focused on the concept of chemoprotection (Shapiro et al., 2001; Bonnesen et al., 2001). DNA protection and enlarging life span assays in animal models are two important *in vivo* insights for evaluation of the health-promoting role of rocket and SF. As extension of healthspan is of critical importance in aging human population, there is a need for screening foods that may support healthy aging. The main objectives of this work were: (i) to analyse the glucoraphanin, SF and total GL content of four accessions of *Eruca sativa*, (ii) to establish the genotoxic and antigenotoxic activities of the *Eruca* material, using the *in vivo D. melanogaster* SMART test, and (iii) to examine the effects of *E. sativa* extracts and SF supplementation in the diet on the life and health span of *Drosophila*.

2. Materials and methods

2.1. Plant material and greenhouse experiments

Seeds of *E. vesicaria* subsp. *sativa* Es1 (commercial variety Sky), Es2, Es3 and Es4 were obtained from Tozer Seeds Lyd (Cobham, Surrey, UK), Faculté des Sciences Agronomiques of Gembloux, Belgium, Botanischer Garten der Universität of Karlsruhe, Germany, and Dipartimento di Scienze Botaniche of Palermo, Italy, respectively. They were germinated in Petri dishes at a temperature of 25 °C for 48 h. Pots were placed under natural light, temperature of 27/18 °C (day/night) and a relative humidity of 50/70% (day/night) in the greenhouse. When the plants reached proper height (8–12 cm), they were transferred to soil.

2.2. Sample preparation

The accessions were harvested once they were ready for human consumption. They were washed with tap water, weighed to assess their biomass, stored at −80 °C and freeze-dried.

2.3. Glucosinolate analysis by liquid chromatography with ultraviolet photometric detection

Freeze-dried leaves of rocket (100 mg) were ground in a Janke and Kunkel (A10 mill, IKA-Labortechnik). The flour was heated at 75 °C to inactivate myrosinase (15 min, 2.5 mL of 70% aqueous methanol). Sinigrin (200 μL, 10 mM) was added as an external standard (Sinigrin hydrate, 85440 Fluka). A second extraction was applied after centrifugation (5 min, $5 \times 10^3 g$) with 2 mL of 70% aqueous methanol. One milliliter of the GL extracts was pipetted onto the top of an ion-exchange column with Sephadex DEAE-A25 (1 mL, 40–125 μm bead size, 30000 Da exclusion limit). Purified sulfatase (75 μL) was added for desulfation (EC 3.1.6.1, type H-1 from *Helix pomatia*, Sigma–Aldrich). Desulfated GLs were eluted with Milli-Q (Millipore) ultrapure water (2.5 mL) and analysed with a 600 HPLC instrument (Waters) equipped with a 486 UV absorbance detector (Waters) at 229 nm. A Lichrospher 100 RP-18 in Lichrocart column (125 mm × 4 mm i.d., 5 μm particle size, Merck) was used for separation and the HPLC chromatogram was compared to the desulpho-GL profile provided by three certified reference materials recommended by U.E. and ISO (CRMs 366, 190 and 367) (Commission of the European Communities, report EUR 13339 EN, 1-75) (Wathelet et al., 1991). The content of GLs was quantified using sinigrin according to the ISO norm (ISO 9167-1, 1992). The total GL content was computed as the sum of all the individual GLs present in the sample.

2.4. Sulforaphane determination by liquid chromatography and mass spectrometry detection (LC–MS)

Freeze-dried leaves (40 mg) of *E. vesicaria* subsp. *sativa* were hydrolysed in phosphate saline buffer (PBS), incubated during 2 h and then centrifuged (13,000*g*, 30 min at 4 °C) to obtain ITCs from GLSs. Supernatant was analysed using liquid chromatography with mass spectrometric detection with positive API-ES (LC/MS) with an 1100 Agilent LC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector and a mass spectrometric detector. SF was monitored using absorbance at 229 nm, and with a selected ion monitoring (SIM) targeted on m/z 178.0. SF quantification was performed by comparing the mass spectrum and the retention time (S8044 Laboratories, Inc., USA) basing on retention time and mass spectrum. A gradient liquid chromatographic separation was performed on a C18–3 μm (150 × 4.6 mm) column, 0.1% formic acid in H_2O and 0.1% formic acid in CH_3CN as mobile phase (flow rate 0.3 mL/min).

2.5. Genotoxicity assays

2.5.1. Strains

Detailed genetic information of the mutations is provided by Lindsley and Zimm (1992). Two *D. melanogaster* strains were used containing genetics markers on the left arm of chromosome 3: (a) *mwh/mwh*, carrying the wing cell marker multiple wing hairs (*mwh*) (Yan et al., 2008) and (b) $flr^3/In(3LR)TM3,\ ri\ p^p\ sep\ bx^{34e}\ e^s\ Bd^S$ ($flr^3/TM3,\ Bd^S$ abbreviated). Being the wing cell marker *flare* (flr^3) (Ren et al., 2007) a zygotic recessive lethal, which is maintained in the strain over the balancer chromosome *TM3*. All experimental flies were reared in a humidified, temperature-controlled incubator at 25 °C and 65% humidity.

2.5.2. Treatment procedure

2.5.2.1. Crosses. One hundred *mwh/mwh* males were mated to virgin females with the genotype $flr^3/TM3,\ Bd^S$. An optimal design requires the double of females than males. Flies are allowed to mate for 3 days to obtain an optimal production of hybrid eggs on the fourth day after mating.

2.5.2.2. Treatments. Genotoxicity and antigenotoxicity tests were carried out as described by Graf et al. (1984, 1998). Flies are allowed to lay eggs for an 8-h period. After 72 ± 4 h, the emergent transheterozygous larvae were washed with distilled water and transferred to treatment vials. Lyophilized leaves of the accessions and SF (Sigma S6317) were dissolved in distilled water at room temperature at decreasing concentrations. For SF treatments, the highest concentration was selected supposing that 100% of the maximum value for the content of glucoraphanin contended in 5 mg/mL (highest concentrations of rocket extract analysed) of vegetal extract was hydrolysed to sulforaphane. Fresh solutions were added to 4 mL treatment vials with 0.85 g of *Drosophila* Instant Medium (Formula 4-24, Carolina Biological Supply, Burlington, NC, USA). Negative and positive controls were